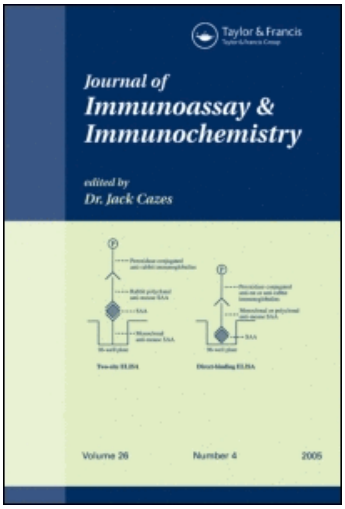

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Beta-Thromboglobulin Radioimmunoassay. A Laboratory Characterization and Evaluation

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BETA-THROMBOGLOBULIN RADIOIMMUNOASSAY

A LABORATORY CHARACTERIZATION AND EVALUATION

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Abstract

Platelets release beta-thromboglobulin from alpha-granules when they are activated by various stimuli. An evaluation and optimization of a radioimmunoassay for beta-thromboglobulin is described. The optimum conditions for the reaction have been characterized, and the use of second antibody and polyethylene

glycol allows completion of the assay within 24 hours. Similar BTG concentrations were obtained using a 1-hour non-equilibration assay but the 1-hour assay was inefficient for processing large volumes of specimens and has the potential for cross reactivity. BTG standards were unstable but the shelf-life was prolonged with aprotinin or by storage at -70°C. Plasma BTG concentration in 80 normal individuals was 28 ± 18 ng/ml. (mean ± 2 S.D.)

INTRODUCTION

During activation of platelets, specific proteins are released from the a-granules. One of these is beta-thromboglobulin (BTG) which can be measued by a radioimmunoassay (1). Preliminary data indicate that BTG concentration is raised in patients with cerebrovascular accidents, prosthetic heart valves, deep vein thrombosis and diabetes mellitus with arterial complications. (2)

Although the BTG radioimmunoassay has been described and used to measure plasma BTG as an indication of platelet activation in vivo, details of the characterization and evaluation of the assay have not been documented. Immunoassay has many experimental variables and meticulous control of these requires definition and understanding of the reaction conditions of the assay, e.g. equilibration time, reversibility of reaction and temperature dependence of binding conditions for maximum discrimination between bound and free ligand. This paper describes an evaluation and optimization of the BTG radioimmunoassay and indicates the rationale for the assay conditions chosen for the measurement of plasma BTG.

In particular, we describe 1) the use of a second antibody with added polyethylene glycol to accelerate the separation of bound ligand

from free ligand, allowing the entire assay to be completed in 24 hours, 2) the stability of standards and labelled ligand, 3) the effect of changes in both standards and labelled ligand on plasma BTG measurement and 4) a comparison of a rapid 1-hour incubation assay with the optimized 24-hour incubation assay.

Materials and Methods

Purified beta-thromboglobulin (3) and rabbit anti-beta-thromboglobulin antibody were generous gifts of Dr. D.S. Pepper (Edinburgh and South-East Scotland Regional Blood Transfusion Centre). Phosphate buffer (0.05M) pH 7.8 with 0.1 M NaCl(PBS)was used throughout the assay.

Plasma for BTG assay was collected into a special anticoagulant mixture to minimize in vitro release. The mixture reported to be most suitable to achieve this consisted of 0.1 ml. ethylene diamine tetra-acetate (Fisher Scientific Co., Toronto, Canada) 0.219 M. 0.1 ml. theophylline (Sigma Chemical Co., St. Louis, Mo.) 30 mM, and 0.1 ml. Prostaglandin E_1 (Sigma Chemical Co., St. Louis, Mo.) 10 uM. (4) The anticoagulant mixture was stored in 12 x 75 mm polypropylene test tubes at -70°C until used. Other investigators have used adenosine instead of Prostaglandin E_1 in the anticoagulant mixture (5).

Preparation of Patient Plasma

Blood samples were drawn with minimum trauma using a 21 G "butterfly" needle and 3 ml. polypropylene syringe. Immediately after insertion of the needle, the tourniquet was released and 2 ml. of blood drawn and discarded to avoid possible contamination of the specimen with tissue thromboplastin. A second 3 ml. syringe was attached and 2.7 ml. of blood was withdrawn slowly and expressed into pre-cooled 12 x 75 mm polypropylene tubes containing 0.3 ml of the anticoagulant and platelet stabilizing mixture (4). These tubes

were stored in an ice-water bath and centrifuged within 1 hour of collection.

Platelet poor plasma was prepared by centrifugation at 2000 g for 60 minutes at 4°C. The middle one-third of the plasma was then removed and deep frozen (-70°C) until assayed.

^{125}Iodine-Labelling of Beta-Thromboglobulin (BTG)

Beta-thromboglobulin was labelled with ^{125}Iodine by the Chloramine-T procedure of Hunter and Greenwood (6). Na ^{125}I, 17 Ci/mg (New England Nuclear, Boston, Mass.) was diluted with phosphate buffer (0.4 mol/litre, pH 7.4) to give 500 uCi per 10 ul. volume. 10 ul Na ^{125}I was added to 5 ug BTG protein in 30 ul of phosphate buffer (0.4 mol/litre, pH 7.4). 10 ul of Chloramine-T (2 g/litre: British Drug Houses, Toronto, Canada) was added, and after 30 seconds at room temperature, 50 ul of sodium metabisulfite (2 g/litre) was added. After addition of 5 ul of potassium iodine (166 g/litre), the labelled ligand was separated from free iodine by use of a column (1 x 25 cm) containing cellulose CF-11, pre-equilibrated with PBS. The column was first washed with twice its volume of PBS to remove unreacted NA ^{125}I. The iodinated BTG was then eluted with 6-8 ml of the same phosphate buffer containing 20 g/litre bovine albumin (Sigma Chemical Co., St. Louis, Mo.) and 20% V/V acetic acid. Immunoreactivity of radio-labelled BTG using excess antibody was 90-95%. The iodinated BTG was diluted in PBS containing 40 g/litre bovine albumin so that 100 ul contained about 1 ng of BTG and yielded about 10,000 counts/min.

Goat anti-rabbit gamma globulin was obtained from Antibodies Incorporated, Davis, Ca. Polyethylene glycol (PEG:M.W. 6000) was obtained from British Drug Houses, Toronto, Canada. Normal rabbit serum (NRS) was obtained from Grand Island Biological Company, Grand Island, N.Y.

Assay Procedure

The following assay procedure was adopted based on the evaluation and optimization of the BTG radioimmunoassay.

Anti-BTG antibody, 100 ul, was diluted in NRS-EDTA phosphate buffer (0.05M phosphate buffer, pH 7.4, with NRS 20 mg/litre and EDTA 18.6 g/litre), to give a concentration that bound 50% of labelled ligand, was incubated at 4°C with 100 ul of buffer standard or plasma in 10 mm x 75 mm plastic tubes for 24 hours. Bound and free moieties were separated by addition of 100 ul of goat anti-rabbit gamma globulin (15-fold dilution) and 100 ul of PEG (100 g/litre). After mixing the contents and allowing the tubes to stand at room temperature 5 minutes, the tubes were centrifuged at 1500 g for 20 min. The supernatant (free) fraction was aspirated and the precipitated (bound) fraction counted.

In the routine assay, first antibody dilution was 1:300,000. This was the experimentally determined antibody dilution that bound 50% of labelled ligand. Plasma samples were assayed undiluted or diluted twofold in PBS with bovine albumin 20 g/litre, and incubated overnight at 4°C with first antibody.

To evaluate a 1 hour non-equilibrium assay, first antibody was diluted with NRS-EDTA phosphate buffer to 1 : 50,000 (experimentally determined to bind 50% labelled ligand in 1 hour), the plasma was assayed undiluted and diluted with phosphate buffer containing 20 g/litre bovine albumin, and incubation with first antibody was for 1 hour at room temperature. Separation of bound ligand from free ligand was by use of a second antibody and PEG.

Standards were prepared by diluting stock BTG solution in PBS (with added bovine serum albumin, 20 g/litre) to give concentrations of BTG of 5, 10, 20, 50 and 100 ng/ml. A zero standard consisting only of phosphate buffer with bovine albumin 20 g/litre was included.

Plasma samples were pooled to create low, medium and high quality control materials. Antibody binding capacity (Ab) and equilibrium constant (K) were determined from a Scatchard plot (7) with computer optimization of upper and lower limits of counts bound, and results were calculated from the expression $L = (Ab/y) - [K^{-1}/(1 - y)]$ where y is bound/total counts and L is total ligand concentration, labelled and unlabelled (8).

Method Development

Antibody Dilution - Anti-BTG antibody obtained from Dr. Pepper was used in this method development. A series of zero-standard tubes were prepared containing labelled ligand diluted to give 10,000 counts/min. and with dilution of first antibody $1:10^3$, $1:10^4$, $1:10^5$, $1:10^6$. After incubation at 4°C for 72 hours, second antibody (anti-IgG antibody) diluted 15-fold was added, the mixture was incubated overnight and after centrifugation, the deposit constituting the bound fraction was counted. The dilution that gave a bound/free ratio of 1 was calculated and was found to be 1 : 300,000. This dilution was selected to study the reaction rate.

Forward Reaction Rate - At this antibody dilution, zero standard tubes were incubated at 4°C for 72, 48, 24, 3 and 1 hour before terminating the reaction by adding second antibody. We found the forward reaction had proceeded to 95% completion by 24 hours.

Reversibility of Binding - Assessment of the reversibility of the reaction was accomplished by the addition of 5000 ng (50 times the highest standard) to zero tubes which had already reached equilibrium with first antibody (72 hrs). The reaction was terminated after an additional 1 hour incubation with second antibody. No labelled ligand was displaced from the initial antibody/antigen complex which implies that the initial equilibrium will not be disturbed when the antibody-bound ligand is separated from the free. This observation

also indicates that increased sensitivity may be obtained by delaying the addition of tracer to the initial incubation mixture.

Temperature Dependence of Equilibrium Constant - Tubes for standard curves were incubated at 4°C, 20°C and 37°C for 24 hours. The equilibrium constant, 12.0 x 10^9 litre/mol, of the present antibody was temperature dependent and was highest at 4°C. At 20°C and 37°C it was respectively 8.00 and 5.00 x 10^9 litre/mol. The reaction is enthalpy driven (H = -14.1 K Cal/mol) with moderate entropy change (ΔS_u = 41.8 K Cal/mol/°K) (9).

Separation System - The effect of second antibody concentration on the separation of free from bound ligand was measured using 100 ul of second antibody diluted 5, 10, 20 and 40-fold. Dilution was tested in 2 extreme situations (10) approximating, respectively, no binding and complete binding of labelled ligand. When labelled ligand in regular amounts (0.1 ng/tube) was mixed with a large excess of unlabelled ligand (500 ng/tube) before addition of first antibody (1 : 300,000) there was effectively no binding of labelled ligand. By contrast, virtually all of the labelled ligand was bound if no unlabelled ligand was added and the first antibody was present at 100 times (1: 3000) the regular concentration. The concentration of rabbit gamma globulin in these two situations was not appreciably different because the buffer contained normal rabbit serum at high concentration.

A second antibody dilution of 1:15 provided maximal discrimination between the upper limit and the lower limit of counts bound. Definition of these limits enabled the observed counts bound to be correlated prior to calculation of the Scatchard plot (9).

Polyethylene glycol (M.W. 6000) at a final concentration of 20 g/litre in the incubation mixture accelerated the precipitation of gamma globulin by anti-gamma globulin but did not precipitate label in the absence of second antibody (11). The time dependence of the second

antibody precipitation in a zero standard tube in the BTG assay with and without polyethylene glycol (PEG) is shown in Fig. 1. In the presence of PEG, precipitation was complete in 5 minutes, whereas in its absence, precipitation was still incomplete at 12 hours. This agent was therefore used in the routine BTG assay with an incubation time of 5 min. before centrifugation. Premixing of PEG (100 g/litre) in equal parts with second antibody (15-fold diluted) conveniently allows addition of 200 ul of the combined reagent to each assay tube and this pre-mixed reagent was stable for 24 hours despite slow development of opalescence (12).

Standard Curve: Definition of Binding Constants - Using first antibody at a dilution of 1 : 300,000, labelled ligand 10,000 counts/min per tube and a range of standards from zero to 100 ng/ml, a standard curve was set up with first incubation 24 hours at 4°C, followed by overnight incubation with second antibody (15-fold diluted) without PEG. In addition, 3 zero standard tubes were set up with increasing labelled ligand amounts of 20,000, 40,000, 70,000 counts/min. per

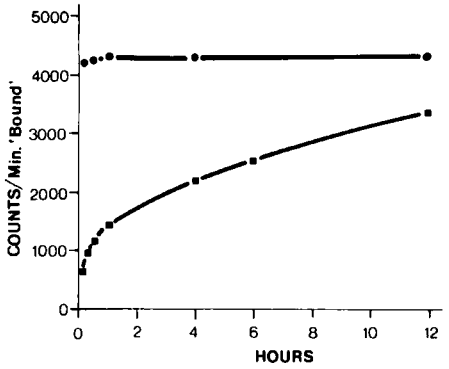

Fig. 1. Time dependence of second antibody precipitation in a zero standard tube in the B-thromboglobulin assay with ● and without ■ polyethylene glycol M.W. 6000, 20 g/litre in final incubation mixture.

tube. These 3 tubes contained the regular mass of labelled ligand present in every standard tube, with additional mass increments of 1, 3 and 6 times the mass present in 10,000 counts/min. The corresponding bound/total counts (y_1, y_3, y_6) read off the standard curve gave results (L_1, L_3, L_6) in terms of unlabelled ligand mass per tube. When divided by 1, 3,6 respectively, these values yielded 1.2, 1.8 and 1.5 ng/tube as estimates of the labelled ligand mass/tube, L*. The coincidence of these values indicated that antibody was not discriminating between labelled and unlabelled ligand.

Sensitivity and Operating Range of Assay - The dilution of the anti-BTG antiserum that bound 50% of the tracer was found to be 1: 300,000. The experimental sensitivity or detection limit at this dilution of antibody was determined by setting up a standard curve with the zero tube replicated 30 times. When no unlabelled ligand was present the sensitivity of the assay expressed as two standard deviations of the bound fraction was 4.02 ng/ml of plasma. The upper limit of the assay at which the bound fraction was twice the non-specific binding was 100 ng/ml when using 100 ul of plasma.

Matrix Effects

1. Plasma Addition - Plasma with low BTG (25 ng/ml) in 100 ul volume was added to a set of standard tubes; to another set of standard tubes was added 100 ul of buffer (Fig 2). The displacement at standard concentration of 12 ng/ml, 24 ng/ml and 50 ng/ml were 27 ng/ml, 26 ng/ml and 25 ng/ml respectively, indicating no significant plasma matrix effect.

2. Plasma Dilution - Three separate samples with different BTG levels were each assayed at 4 different dilutions. The results are shown in Fig. 2 and are identical when corrected for dilution within the confidence limits of the assay.

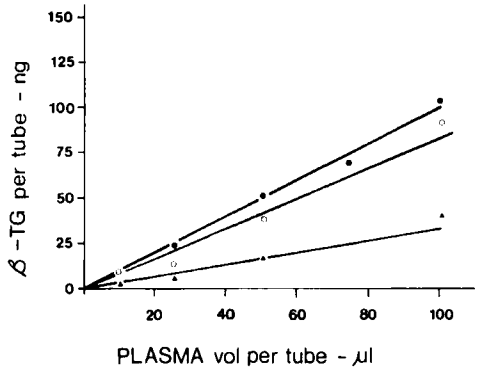

Fig. 2. Three different plasma samples were each diluted 1:2, 1:4 and 1:10 and BTG level of each sample and dilution was determined. This figure shows the effect of plasma dilution plotted as BTG, ng per tube, against plasma volume, ul per tube.

3. Sporadic Matrix Effect - In order to detect sporadic matrix effects, all samples were routinely assayed in duplicate at each of 2 dilutions, 100 ul and 50 ul per assay tube. In 200 samples assayed, no volume dependent discrepant results were apparent.

4. Matrix Effects due to Label - Quality control samples were compared using freshly prepared labelled BTG and labelled ligand. stored for 6 weeks at -70°C. A significant increase in assayed values was noted when the stored tracer was used.

Immunoreactivity using excess antibody of the stored tracer was 70%, compared to 90% for freshly labelled BTG. Chromatography using an 85 x 2.5 cm column packed with Séphadex G75 revealed three peaks (Fig 3). The major peak had an immunoreactivity similar to freshly prepared labelled ligand. The second major peak was completely removed by anion exchange resin (AG1-X10) suggesting it consisted of protein aggregates.

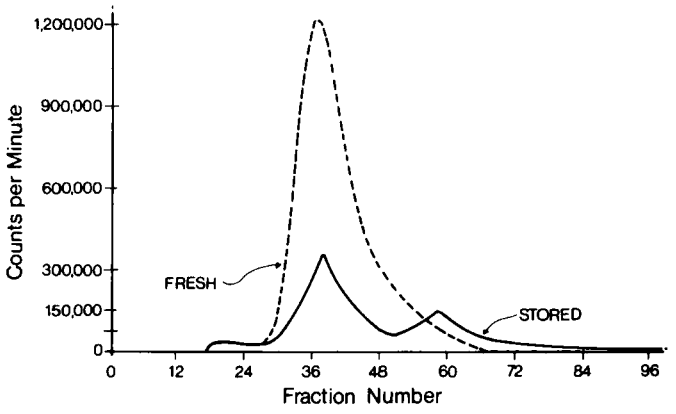

Fig. 3. Comparison of chromatographic pattern of fresh and stored ^{125}I-BTG tracer using G-75 sephadex column. Eluting fluid 0.05 mol/litre phosphate buffer, pH 7.4, containing bovine serum albumin 10 g/litre.

5. Cross-reactivity - The following antigens were examined for cross reactivity: fibrinogen, albumin and platelet factor 4. They showed no cross reactivity with BTG antibody, confirming previous observations (4)

Precision

Within-run precision, measured on 3 plasma samples, assaying each sample 20 times within one run is shown in Table 1. In the same

Table I. Precision of plasma βTG measurement (n = 20)

	WITHIN-RUN PRECISION			BETWEEN-RUN PRECISION		
	Low	Medium	High	Low	Medium	High
$\bar{x}$ ng/ml	28.1	57.0	109.0	33.1	69.1	115.0
S.D.	2.35	4.92	15.2	6.3	13,9	14.8
* CV %	10%	9%	14%	19%	20%	13%

* CV = Coefficient of variation

table, between-run precision for low, medium and high concentration control sera based on 20 runs during 6 months is shown. The within-run precision is 10% (1CV) for low and medium concentration, and 14% (1CV) for high concentration. The between-run precision during 6 months is 13-20%.

1-Hour Non-equilibrium Assay

A rapid 1-hour non-equilibrium assay using a 1: 50,000 dilution of antibody was compared to the optimized 24 hour assay described here and BTG concentrations were not significantly different (Table II).

Effect of Heparin on Assay

The effect of heparin on the assay system was investigated. Heparin concentrations greater than 10 U/ml interfered with the non-equilibrium 1-hour assay because it assayed as equivalent to 10-30 ng/ml BTG but did not interfere with the assay using the 24 hour equilibrium incubation. Although the non-equlibrium interference effect by heparin is not clinically important, as such levels of plasma heparin are not usually encountered in patients, the observation illustrates the benefit of using an optimized assay to avoid chemical or drug interference.

Table II. Comparison of a 1-hour assay and 24-hour incubation assay on 6 plasma samples

Sample	1-hour	24-hour
1	8.7	13.4
2	19.5	18.4
3	44.3	41.0
4	18.1	18.3
5	27.2	28.1
6	19.6	19.1

Stability of BTG Standard with Storage

The BTG was dissolved in PBS with bovine albumin 10 g/litre and diluted to give stock aliquots of 5000 ng/ml of standard which were stored at -70°C.

Working BTG standards were stable during 7 days storage at 4°C but not beyond this time. Standards stored longer gave a lower reading, with up to 50% loss after 10 days of storage in the absence of aprotinin. Addition of 25 U/ml of aprotinin extended stability of standards up to 1 month of storage at 4°C (Table III). Standards stored at -70°C were unaltered antigenically up to 12 months. These observations suggest that instability of standards is related to proteolytic action and that BTG can be protected by storage with aprotinin.

BTG Level in Normal Individuals

In 80 healthy individuals (40 female and 40 males aged 21-45 years, mean 28), mean plasma BTG level was 28 ± 16 ng/ml (range 9-45 ng/ml) with no sex or age difference and are similar to a pre-

Table III. Stability of βTG standard stored at 4°C with aprotinin

* Conc^{n} ng/ml \ Time	Fresh †	3 Weeks †	4 Weeks †
10	8.6	9.7	11.8
20	18.9	19.6	23.6
50	52.9	49.4	56.9
100	96.3	98.2	109.5

• Aprotinin Concentration 25 U/ml

• Expected βTG concentration

† Measured βTG concentration at the time stated

viously published report (13). In 6 normal individuals, no variation in plasma BTG concentration was noted on sampling 10 times over 15 minutes from the same venepuncture site. Samples obtained at intervals of 1 week showed little variation in BTG concentration and a similar lack of variation was found in samples taken 1 month apart for 3 months. Mean platelet BTG content in 10 healthy males with mean platelet count of 300 x 10^9/L was 25 ng/10^6 platelets (range 19-30 ng/10^6).

DISCUSSION

The optimization of a radioimmunoassay for beta-thromboglobulin, a platelet specific protein, is described. Separation of bound ligand from free ligand was by second antibody with added PEG. In our development of the BTG assay we have shown that the antibody-ligand reaction had reached equilibrium by 24 hours incubation at 4°C. The addition of PEG to the second antibody reduced the time of incubation for separation of bound from free from 24 hours to 5 minutes. Without PEG, separation was still incomplete at 12 hours incubation. This finding is similar to that described for the assay of serum ferritin (12). Hence with the present assay, BTG determination was complete after a total of 24 hours incubation.

As clinical usage requires rapid assay results, we have compared a non-equilibrium 1-hour incubation assay to the 24-hour incubation assay. The 1-hour assay gave similar results to the 24-hour assay. However, the non-equilibrium system does require an antibody concentration 10-20 times that of the equilibrium system. Another potential disadvantage of the rapid non-equilibrium assay is the possibility of interference by drugs, as demonstrated by high concentrations of heparin.

Working BTG standards, once prepared, have limited stability at 4°C, with a shelf-life of 5-7 days. However, storage at -70°C

increased the shelf-life to at least 6 months. The addition of aprotinin at a concentration of 25 U/ml prevented loss of BTG standards stored at 4°C for up to 4 weeks. These observations suggest that BTG decay on storage at 4°C is related to proteolytic activity present in the BSA preparation (14). The ability to store these standards longer led to an economy of consumption of this platelet-specific protein.

The 24-hour equilibrium assay has been in operation in our laboratory for one and a half years and has shown itself to be robust. Plasma from various groups have been assayed with little difficulties encountered.

The mean plasma BTG level in a group of healthy individuals was 28 ng/ml $\pm$ 16(2 S.D.) and did not vary,and mean platelet BTG content was 25 ng/10^6 platelets. Plasma BTG has a short in-vivo half-life of 100 minutes (15) and because plasma BTG concentration in normals is stable, a constant level of platelet utilization may be postulated.

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